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Defining the biosecurity risk posed by transported soil: Effects of storage time and environmental exposure on survival of soil biota

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Abstract

Soil frequently occurs as a contaminant on numerous sea, land and air transport pathways. It can carry unwanted invasive species, is widely recognized as a biosecurity risk, and is usually strictly regulated by biosecurity authorities. However, little is known about relative risk levels between pathways, thus authorities have limited capability to identify and target the riskiest soil pathways for management. We conducted a an experiment to test the hypotheses that biosecurity risks from soil organisms will increase both with declining transport duration and with increasing protection from environmental extremes. Soil was collected from two sites, a native forest remnant and an orchard, and stored on, in and under sea containers, or in cupboards, and assayed after 0, 3, 6 and 12 months for bacteria, fungi, nematodes and seeds.

Results showed that viability of *Pseudomonas* spp., bacteria, nematodes and plants declined over 12 months, irrespective of soil source. Also, mortality of most biota was higher when exposed to sunlight, moisture and desiccation than when protected. However, bacterial and fungal numbers were higher in exposed environments, possibly due to ongoing colonization of exposed soil by airborne propagules. The results were consistent with our observations of organisms in soil intercepted from airports and sea ports, and indicated there is potential to rank risks from transported soils based partly on transport duration and environmental exposure. This would help authorities to optimally allocate management resources according to pathway-specific risks.

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Keywords
Species invasion, pathways, risk analysis, trade, tourism

Introduction

Protecting primary industries and native flora and fauna from invasive alien species (IAS) is critical to all nations' economic welfare and biodiversity (e.g. Pimentel et al. 2001; Early et al. 2016, Paini et al. 2016). Research that allows regulatory authorities to make rational evidence-based decisions about biosecurity risks (pathways and pest species) posed by arrivals of tourists, freight, machinery and biological material is essential to these aims. Soil frequently occurs as a contaminant on transported items, is widely recognised as a vector for non-native species, and is often the target of management practices that aim to minimise the spread of IAS (Catley 1980, Sequeira and Griffin 2014, Eschen et al. 2015, Migliorini et al. 2015). However, knowledge of the biosecurity hazards that can be vectored in transported soil, their relative survival rates on different pathways, and their establishment probabilities is currently insufficient to support optimal policy and management decisions (Brunel et al. 2014, Hulme 2015, Singh et al. 2015).

Comparisons between studies suggest that biosecurity risks from transported soil will vary depending on a range of biotic and abiotic factors including the taxa and life stages present, duration of transport, and exposure to environmental extremes during transport. For example, previous research suggests soil transported by sea, which will have had relatively long transport durations, contains fewer organisms than soil transported by air: Construction vehicles shipped by sea contained 0.002 nematodes/g (Hughes et al. 2010) and soil from sea containers contained 0.07 nematodes/g (Marshall and Varney 2000), while soil from air passengers' footwear contained 41 nematodes/g (McNeill et al. 2011). Similarly, 11% of seeds from construction vehicle soil were viable (Hughes et al. 2010) c.f. 69% in footwear soil (McNeill et al. 2011). McCullough et al. (2006) recorded a lower diversity of organisms from soil on cargo arriving by sea and air than from soil in luggage, and a recent study (McNeill et al. unpublished) recorded generally lower incidences and counts of organisms/g of soil from sea containers compared to air passengers' footwear (McNeill et al. 2011).

Marshall and Varney (2000) suggested that soil organisms transported on the external surfaces of sea containers might suffer high mortality rates due to exposure to solar radiation and sea water. As transport duration is typically longer by sea than air, McNeill et al. (2011) further postulated that incidences and counts of soil taxa could also be influenced by the time elapsed from when imports become contaminated with soil, and when the soil is sampled to assess biosecurity hazards (hereafter referred to as 'soil age'). Unfortunately, observational studies can generally provide only imprecise estimates both of soil age, and of the environmental conditions that soil has been subjected to during transport. For example, container ships may visit several ports en-route to a destination, with soil contamination potentially occurring at any port

where containers are unloaded and reloaded. Moreover, the route that a sea container takes to New Zealand is often only traceable to the last two ports. Similarly, McNeill et al. (2011) could only roughly estimate the age of soil on air passengers' footwear by surveying passengers. Thus, controlled experiments offer better opportunities to measure factors influencing survival of soil biota.

Soil origin is also likely to influence the species that occur in transported soil (Fierer et al. 2007), and will be especially relevant to whether a particular high risk pest species with a restricted geographical distribution could be present. However, there is little published evidence to suggest that soil transported from different locations should show major community differences at higher taxonomic levels (Chu et al. 2010, Decaens 2010, Kuske et al. 1997). For example, McNeill et al. (2011) recorded bacteria, fungi, nematodes, seeds and arthropods in soil samples originating from a diversity of international locations. In addition, for reasons previously explained, the origin of transported soil is frequently impossible to identify and cannot be used for gauging biosecurity risks. Thus, our study focused on the roles of soil transport duration and storage method, rather than soil origin, on survival of soil biota. Nevertheless, our experiment included soil from two contrasting sites to check our assumption that soil organisms from different locations will show similar responses to transport duration and storage method.

Understanding the effects of environmental conditions and transport duration on organism survival is important for developing robust biosecurity risk assessment processes (World Trade Organisation 2010; Burgman et al. 2014). However, such knowledge is lacking for soil organisms (Singh et al. 2015), hence our replicated experimental study asked how different storage conditions and transport durations affect soil biota. Soil was collected, subjected to treatments that simulated differing conditions during transport, and subsampled throughout 1 year to monitor changes in soil organism incidence and abundance.

The results increase knowledge of how environmental exposure and transport time influence soil biota survival, inform pest risk assessments, and help prioritize risks from soil that occurs on different transport pathways. This will assist quarantine authorities to target management resources at the highest risks to improve biosecurity protection without additional cost.

Methods

Soil was collected on 23 August 2011 (winter) from two sites in Canterbury, New Zealand: A regenerating native forest reserve in Prices Valley, Banks Peninsula (43°46.014'S, 172°42.840'E) (soils 1-3); and an organic orchard at Lincoln (43°39.048'S, 172°27.354'E) (soils 4-6). The forest reserve was bounded by farmland and a road, and was fenced to exclude livestock. Soil was taken from three different locations at each site, and each of these six samples were treated separately throughout the experiment. In the native reserve, the three locations were approximately 10 m

apart and, in the organic orchard, the locations were approximately 5 m apart. At each location within a site, six spade square soil samples (14 cm × 14 cm) were taken to a depth of 5 cm at six randomly chosen points within a 3 m radius of an arbitrarily designated central point. Prior to extracting each spade square, vegetation was cut to ground level with scissors and loose litter was removed. The six spade squares were mixed in a stainless steel tray and transferred to a plastic bag. The spade was cleaned with 70% ethanol both between each site, and between each location within sites. Disposable laboratory gloves were worn at all times, and changed between sites.

In the laboratory, the soil was screened with a 10 mm sieve and a subsample was taken for chemical analysis, and for counting microbes and nematodes. The remaining soil from each site (n = 2) and location (n = 3 per site) was divided amongst stainless steel steam trays (external dimensions c. 400 mm × 240 mm × 50 mm; internal dimensions 300 mm × 200 mm × 50 mm), in which twenty 4 mm diameter drainage holes had been drilled into the base, then allocated to treatments (c. 700 g of soil per tray). The soil was spread evenly onto the tray surface and gently pressed with a stainless steel pan to lightly compact it.

Treatments

Each of the six soil samples was divided among the same eight treatments, which are summarised in Table 1 and described below. A data logger was allocated to each treatment to record temperature for the duration of the experiment. Treatments were divided between four cupboards and four sea containers. The four cupboards were placed in an unheated shed. The sea containers (3 m length × 2.4 m height × 2.5 m width) were cleaned with high pressure water, transported to an outdoor concrete pad at Lincoln (S43.6279, E172.4704), and situated c. 2 m apart.

Cupboard treatment

Six uncovered trays (two soil sources × three locations) containing soil were placed inside each of four cupboards, which were located indoors and kept at ambient temperature. Thus, these samples were protected from sun, wind and rain, and were expected to experience less temperature variation compared to samples assigned to the sea container treatments. There was low potential for additional organisms to disperse to these samples.

Sea container treatments

Six uncovered trays containing soil were placed in locations in, on and under each of four sea containers. The six samples placed within each container were protected from

Table 1. Summary of soil samples, treatments and assays to investigate survival of soil biota (bacteria, fungi, nematodes and plants).

Container	Treatments	Sites	Locations	Samples	Bioassay times
Sea container 1	Top, Inside, Under	2	3	6	4
Sea container 2	Top, Inside, Under	2	3	6	4
Sea container 3	Top, Inside, Under	2	3	6	4
Sea container 4	Top, Inside, Under	2	3	6	4
Cupboard 1	Inside	2	3	6	4
Cupboard 2	Inside	2	3	6	4
Cupboard 3	Inside	2	3	6	4
Cupboard 4	Inside	2	3	6	4

sun, wind and rain. They were expected to experience less temperature variation and lower rates of invasion by additional soil organisms. The six samples placed on top of each container were exposed to UV, wind and rain. They were expected to experience maximal temperature variation, and high rates of invasion by additional soil organisms such as windborne microbes and seeds. The six trays placed underneath each container were positioned in the fork lift cavities. They were protected from direct sunlight, and rain, but were exposed to wind and had potential to become wet. They were expected to experience moderate temperature variation and high rates of invasion by additional soil organisms such as windborne seeds and perhaps some arthropods.

Untreated controls

The sites from which the soil was originally sourced were resampled on the same dates the container samples were assayed on 28 November 2011 (spring), 27 February 2012 (summer) and 27 August 2012 (winter). The top 5 cm of soil was sampled from six randomly chosen points at each site (n = 2) and location (n = 3 per site). Soil from six spade squares per location was mixed in a stainless steel tray, then assayed in the same way as the sea container and cupboard treatments. These samples are hereafter referred to as 'fresh soil'.

Bioassay times

The incidence and abundance of soil organisms persisting within each tray was assayed on day 1 (23 August 2011, winter), then after 3 months (28 November 2011, spring), 6 months (27 February 2012, summer) and 12 months (27 August 2012, winter). This coincided with sampling of fresh soil.

Each tray was subdivided into five equal areas. At each bioassay time, one c. 20 g subsample of soil was taken from a predefined location within each of the five areas,

thereby providing c. 120 g of soil from each of the six trays per treatment for each assay. However, loss of soil from the trays situated on top of the sea containers meant that the amount collected for the 6 and 12 month bioassays had to be reduced.

Organisms assayed

Soil organisms were assayed as previously described in the study of soil on international air passengers' footwear (McNeill et al. 2011), then identified to the extent achievable with the resources and taxonomic expertise available. Assays included culturing of all culturable bacteria, *Pseudomonas* spp. bacteria, and fungi. *Pseudomonas* spp. were chosen as model bacteria for isolation because several species are important plant pathogens (Silby et al. 2011) and are categorised as regulated species by New Zealand's biosecurity authority, the Ministry for Primary Industries.

Counts of bacteria and fungi

Depending on the weight of each sample, a subsample of 1.05 g to 21.55 g of soil was taken to estimate bacterial and fungal densities (colony-forming units per gram, CFU/g). Each sample was diluted 10-fold using 1% peptone and sonicated for 3 minutes to facilitate mixing. Serial dilutions were plated onto three different media: 10% tryptic soy agar plates with 100 ml/L cycloheximide to determine total bacteria counts; water agar containing 100 mg/L streptomycin for total fungi counts; and Oxoid *Pseudomonas* agar, supplemented with Oxoid CFC (cetrimide 10 mg/L, fucidin 10 mg/L and cephalosporin 50 mg/L) to select for pseudomonads. Fungi were enumerated by serial dilution plating onto water agar containing 100 mg/L streptomycin. For bacteria, plates were incubated at 20 °C (light: dark, 0: 24) and colonies were counted after 7 days. For fungi, the plates were incubated at 20-25 °C and colonies were counted after 10 days. Counts for bacteria and fungi were taken from 186 of 192 samples. It was not always possible to count every plate due to the presence of high concentrations of bacteria or fungi. In these circumstances, experience was used to determine the best dilution result to use.

Nematodes

The amount of soil used for nematode extraction was 100 g for fresh soil, and ranged from 11.34 g to 25.3 g for soil subsampled from the trays, depending on the amount of soil available. Nematodes were extracted from the samples taken on 23 August 2011 following the method of Bell and Watson (2001). This used the Whitehead tray extraction method whereby each sample was placed on two-ply tissue paper, supported by two layers of nylon gauze within a shallow tray to which 500 ml of tap water was

added. The tray was left for 72 hours, after which the liquid was poured into a 1 L plastic beaker, left to settle for 4 hours, then gently reduced to 100 ml volume by removing the supernatant. The 100 ml samples were transferred to 100 ml plastic beakers and allowed to settle for 4 hours before reduction to a final volume of 20 ml. Nematodes were counted in a Doncaster dish (Doncaster 1962), to provide a total nematode count (fungal, bacterial, omnivore, predator and plant parasitic). For samples collected directly from the field sites, a quarter of the sample was counted then multiplied by four to give the estimated total. For those subsamples collected after 3, 6 and 12 months, all the nematodes extracted from the soil were counted. Plant parasitic nematodes were identified to genera based on the keys of Siddiqi (2000) for Tylenchida, and Bongers (1994) for other groups. The plant parasitic nematodes (PPN) were further identified, counted and assigned either to the Criconematidae family, or a plant parasitic genus (e.g. *Helicotylenchus*, *Heterodera*, *Paratylenchus* and *Pratylenchus*).

At the 3 month assay, nematode extraction employed both the Bell and Watson (2001) method and a misting method (Seinhorst 1950; De Waele et al. 1987). The misting method allowed faster throughput of multiple samples. Analysis comparing the two extraction methods on the 24 cupboard samples found no significant difference in nematode yields ($P = 0.724$ for the log total nematodes, and $P = 0.211$ for the the log plant parasitic nematodes) (L. Aalders, unpublished data). Therefore, the misting method was used to extract nematodes from subsequent samples. The misting method involved placing c. 25 g of soil in a mistifier funnel and misting for 30 s every 5 minutes for 72 hours at a water temperature of 20 °C. The mistifier funnel system consisted of a plastic tube (75 mm internal diameter), positioned vertically with a plastic mesh base (1 mm aperture) on which two layers of paper tissue (Kimwipes™, Kimberley-Clark Worldwide Inc.) supported the soil sample. This tube sat on top of a plastic funnel. The water from the overhead mister washed the nematodes through the soil and into a glass test tube. Another c. 20 g of soil was oven dried at 80 °C for 48 hours to measure soil moisture.

To enable comparison across treatments and with previous studies (e.g. McNeill et al. 2011), results are presented as nematodes per g of collected soil, rather than per g of dry soil. Soil samples from which nematodes had been extracted were then used to assess if the soil contained viable seeds (see below).

Plants

Because visual searches are an imperfect method for detecting all seeds in soil samples (McNeill et al. 2011), the soil that had been used for nematode extraction was also used to determine the number of viable seeds using germination tests. After the 72 h nematode extraction, the soil from each sample was laid in a 5 mm thick layer on a paper towel over a wet medium (potting mix) for small samples (<25 g), or on a layer of towels in a small metal tray for the larger samples (100 g). The soil was kept moist under natural light in a quarantine glasshouse (15–35 °C) for up to 12 weeks, and ob-

served 1–2 times per week for germinated seedlings. Seedlings were transplanted into sterile potting mix 1–2 days after emergence and grown on for identification to the lowest possible taxonomic level.

Aids to identification were a combination of web-based keys (e.g. http://www.efloras.org) and published literature on New Zealand native and introduced species (Webb et al. 1988; Edgar and Connor 2000; Champion et al. 2012; James et al. 2012).

Temperature

Temperature and humidity were recorded in each cupboard, and temperature on the top, inside on the floor, and under each sea container. The loggers located on the top of containers were housed within Stevenson-type screens. In addition, two extra loggers recorded humidity (and temperature) inside and under one of the containers. Temperature and humidity data were recorded either every 30 or every 60 minutes using Tinytag™ loggers (Gemini Data Loggers Ltd, Chichester, UK).

Soil chemistry

Soil for chemical analysis was collected at the start of the experiment by taking five 7.5 cm diameter × 5 cm deep cores from each location at each site. Any vegetation was removed with a box cutter. The cores from each location were hand mixed then bagged prior to being forwarded to a soil analysis laboratory. Soil pH, P (Olsen phosphate), K, sulphate sulphur (S SO_4), organic sulphur, total phosphorous, Cu, Co, Fe, Mn, Zn, organic carbon and organic matter were quantified. Soil was classified using Landcare Research's S-map series (http://smap.landcareresearch.co.nz/) (accessed 01 September 2016).

Analysis

Data analysis methods are briefly described here, and full details are given in the Suppl. material 1. Analysis was carried out using a Latin square design, blocked by treatment (cupboard, sea container, fresh) and site (forest, orchard), which allowed measurement of: variation between and amongst cupboards and sea containers; and interactions between site and treatment (e.g. soil with high organic matter content might dry more slowly, thus enhancing organism survival). Obtaining soil from three locations per site enabled comparisons between stored soil (treatments) and fresh soil (untreated controls).

This design has split-plot elements (the site effect is not replicated), nested random effects (soil location nested within site), crossed random effects (soil location nested within site is crossed with storage treatment location), and longitudinal measures (four repeated measurements in time). The null hypothesis was that there was no difference

between the treatment levels upon the effect of time upon the response variables. If the hypothesis was rejected for the response variables, then we explored possible relationships between temperature, treatment and the response variable.

The statistical model that we fitted used the following template.

$$f_L(y_{ijkmt}) = \beta_0 + \omega_i + f_T(t, \tau_m) + \gamma_i + \delta_j + \psi_k + \varepsilon_{ijkmt} \qquad (1)$$

Here,

y_{ijkmt} is the response variable at duration t from soil location j within site i stored in site k under treatment m;

$f_L(\ldots)$ is a selected transformation of the response variable, usually a natural logarithm;

β_0 is a constant intercept for the response variable;

t refers to the duration of storage;

τ_m refers to the treatments (m = 1,…,5)

$f_T(t, \tau_m)$ is some smooth function of the duration and the treatment that is constrained so that all treatment effects are identical at t = 0;

ω_i is a fixed effect representing site (forest / orchard);

$\gamma_i \sim N(0, \sigma_\delta^2)$; i = 1, 2 is the site random effect, which may be confounded with ω_i;

$\delta_i \sim N(0, \sigma_\delta^2)$; j =1, …, 6 is the location random effect, with 3 from each site;

$\psi_i \sim N(0, \sigma_\delta^2)$; k = 1, …, 9 is the storage treatment location random effect, where locations 1 – 4 are sea containers (corresponding to treatments 1 – 3 only), 5 – 8 are cupboards (corresponding to treatment 4), and 9 is the fresh soil treatment; and $\varepsilon_{ijkmtt} \sim N(0, \sigma^2)$ is a random error.

It is reasonable to expect that the response variables will vary with site, so they should be included in the model, as ω_{ii}. However, in order that the three locations within each site not be considered as independent realizations of the predictor variable, we included site as a random effect in the model (γ_{ii}). Including site as a random effect induced the needed correlation between samples from the same site to reflect the design. The soil samples were taken from two sites only, so any test of soil origin, or factors that interact with soil origin, are pseudo-replicated. The tests can not be interpreted as representing all possible soil samples from forests or orchards, although see Discussion. It was sometimes necessary to include those terms in the model as fixed effects in order to accurately capture the variability (see online materials).

This model was applied to seven response variables, namely (i) relative bacteria count per unit soil volume, (ii) fungi, (iii) *Pseudomonas*, (iv) nematodes, (v) plant seeds corrected for soil volume (vi) plant species corrected for soil volume and (vii) the raw number of plant species not corrected for soil volume. A similar model was applied to the response variable defined as the binomial proportion of nematodes that were plant-parasitic nematodes, for which we used only the observations that had non-zero counts of nematodes.

The fixed effects were tested using likelihood ratio tests, with the cut off for statistical significance set at 0.01 in order to loosely compensate for the multiplicity of tests. The

random intercepts were not tested; they were included in order that the model faithfully represent the experimental design. Initially a full model was fitted, and diagnostic graphics were checked. The full model included treatment, site, and storage duration as a linear effect. The outcome of the check was generally the selection of some form of transformation for the response variable, e.g., natural log. We next applied backwards elimination to assess whether site should be retained as a fixed effect, noting that the site fixed and random effects are confounded. A backwards elimination approach was then applied to see how duration should enter the model, including: not at all, as a linear term, as a quadratic term, and as a constrained two-knot spline, with knots set at 3 months and 6 months, which is equivalent in degrees of freedom to a cubic polynomial function, but performed better visually. Invariably, the most complex temporal model was selected by the test. Duration was treated using cubic splines, because extrapolation using the cubic polynomial was unrealistic, and using a category for duration ignores the relative location of the observations in time. For example, the first three measures were separated by 3 months whereas the last measure is after 6 months. The best model was then assessed using graphical diagnostics, and adjustments made if needed. The fitted models were then overlaid upon scatterplots of the data that were augmented with smooth means, as a visual aid to the testing and modelling process.

Although it was commonly true in the analyses that the model assumptions were ratified by graphical diagnostics, sometimes the distribution of the residuals was a little more skewed than symmetric. In these instances, we were reasonably confident that the sample size was sufficiently large that the Central Limit Theorem would hold, and that the test assumptions would be robust to the departure from the nominal assumptions.

The following reasoning for assessing the importance of interactions between random effects was used. There are no reasons to assume that the random effects would interact with duration (see above) and beyond the effects of the interaction of treatment with duration, so the random interactions were not formally tested. However, graphs were constructed to act as diagnostics for this assumption. If there was an important interaction between the random effect and duration, then we assumed that it would be visible in these graphics. In cases where this occurred, we formally tested the extended term using a whole-model likelihood ratio test, and if it was significant, repeated the fixed effects test suite with the new random effects structure. In no case did the test of the fixed effects alter from the original result in any important way; hence, these results were not pursued further. Interactions between fixed and random effects were not tested further.

If the estimated soil location random effect was non-zero, then the effects of soil chemistry upon the response variable was informally assessed by examining scatterplots of the estimated soil location random effects against the soil chemistry variables. More formal analysis did not seem appropriate owing to the large number of potential soil variable predictors. These results are not included here.

If the interaction between treatment and duration was statistically significant, then further analysis was undertaken to assess the effect of temperature. The analysis was performed by taking the temporal differences of the response variable, or transformations of the response variable where appropriate (e.g. natural log for count data and empirical

logit for proportion data) and assessing the effect of the interaction of duration with both treatment and temperature upon the change from assay time to assay time.

All analyses were carried out using the open source statistical environment R (R Core Team 2014). We obtained 95% confidence intervals of the estimated random effects (not shown here), using a parametric bootstrap algorithm that is provided by the lme4 package (Bates et al. 2014).

Results

Earthworms and arthropod larvae (e.g., grass grub, *Costelytra zealandica*) were observed in the soil during its initial collection. Some were inadvertently transferred to the trays, but none were alive in the 3 month assay. For the trays located under the sea containers, colonisation by spiders occurred within 3 months, a dead bird was found in one tray after 12 months, and rodent droppings were observed at intervals throughout the experiment.

Full results from the statistical analysis are in the Suppl. material 1, and are summarised here as follows. Each response variable is presented with a graphical summary that plots duration against the raw data with smoothed means, constructed using a loess algorithm, augmented by dashed lines that represent the best-fitting statistical model. In each case, the results are contrasted with results from the fresh soil that was assayed at the same time.

For all response variables, the most complex model with duration was required. Sample site was statistically important for several variables (namely fungi, nematodes, plant count and plant species count, see online materials).

Bacteria

Modelled and mean observed changes in bacteria across time are presented in Figure 1. The fresh soil retained high counts throughout the 12 months of the study. The cupboard and inside-container treatments had similar trajectories for both forest and orchard soil, dropping in the 12 months to a tenth of the original count or less. Soil located on top of the sea containers retained the initial high counts, and in some cases had levels higher than fresh soil. Soil located under the container suffered a short-term drop, but returned to the same levels as the container-top soil and the fresh treatment. There was no detectable difference between soil sites.

Pseudomonas species

Modelled and mean observed changes in *Pseudomonas* spp. counts across time are presented in Figure 2. Counts from fresh soil remained reasonably constant throughout the 12 months. For stored soils, mortality was highest in cupboards and on top of containers

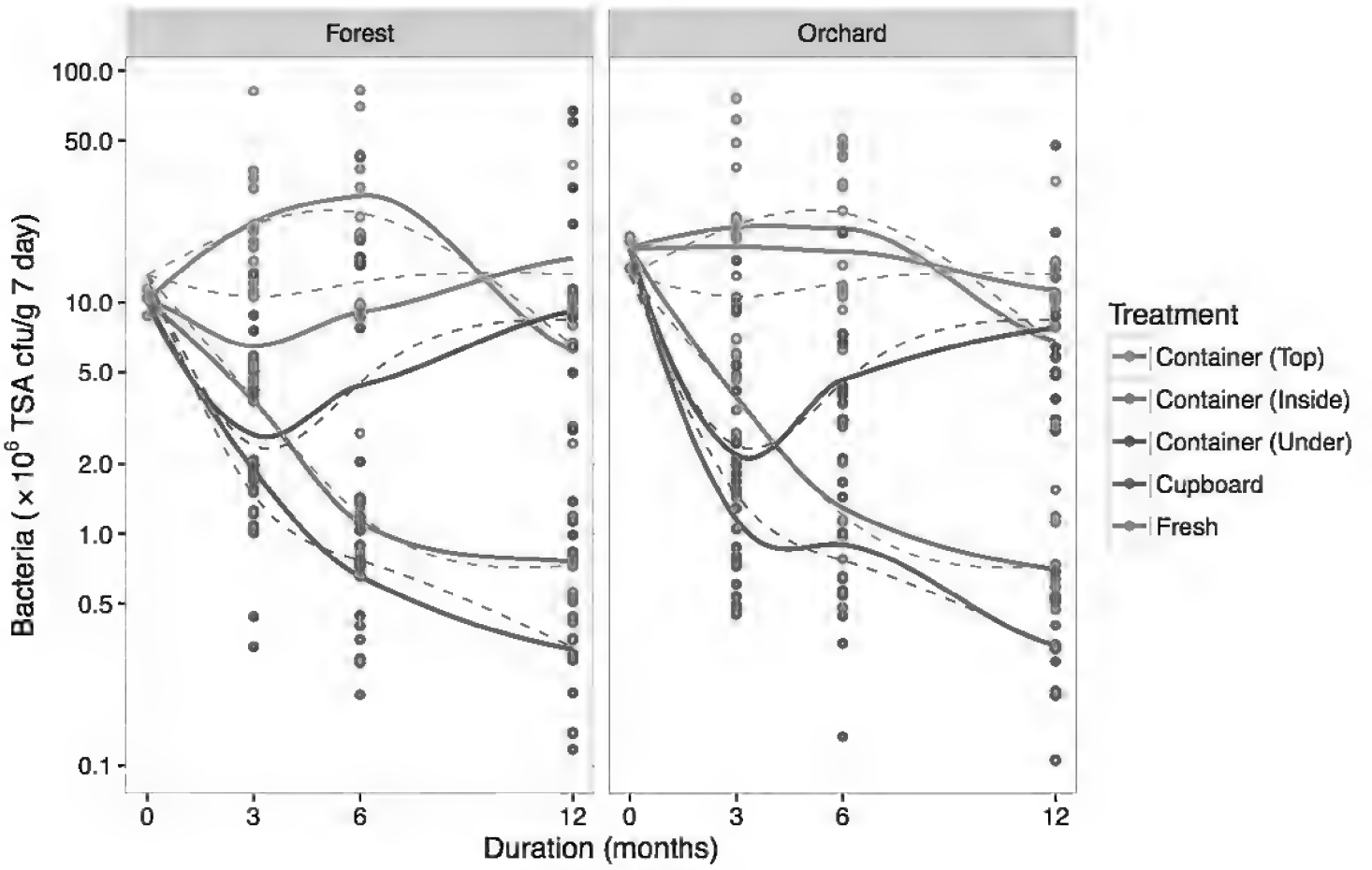

Figure 1. Scatterplot of mean bacterium counts (cfu/g) at 0, 3, 6 and 12 months measured in soil recovered from on, in and under four sea containers and inside four cupboards. Solid lines show the smoothed means and dashed lines predictions from the preferred model.

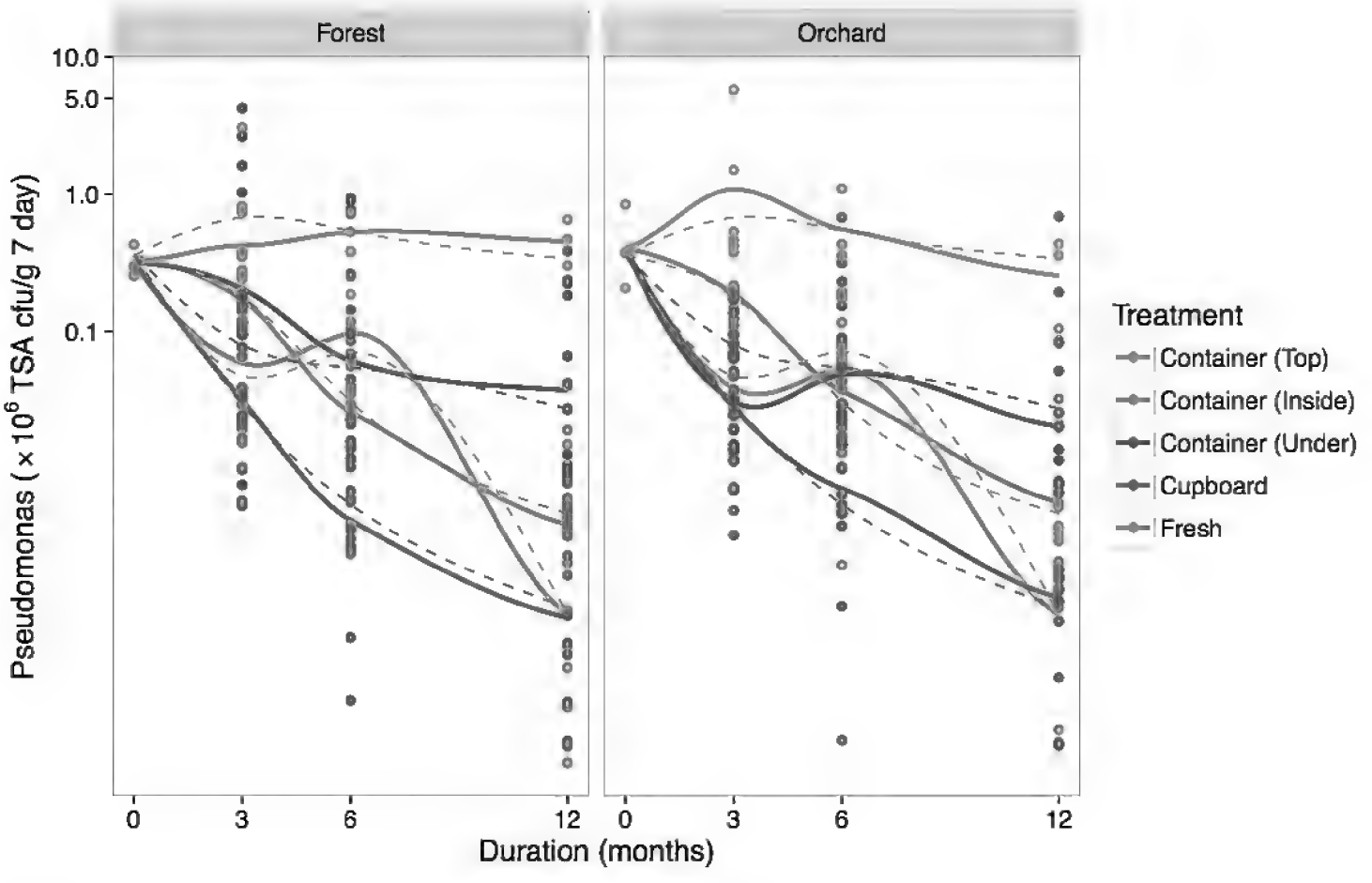

Figure 2. Scatterplot of mean *Pseudomonas* spp. counts (cfu/g) at 0, 3, 6 and 12 months measured in soil recovered from on, in and under four sea containers and inside four cupboards. Solid lines show the smoothed means and dashed lines predictions from the preferred model.

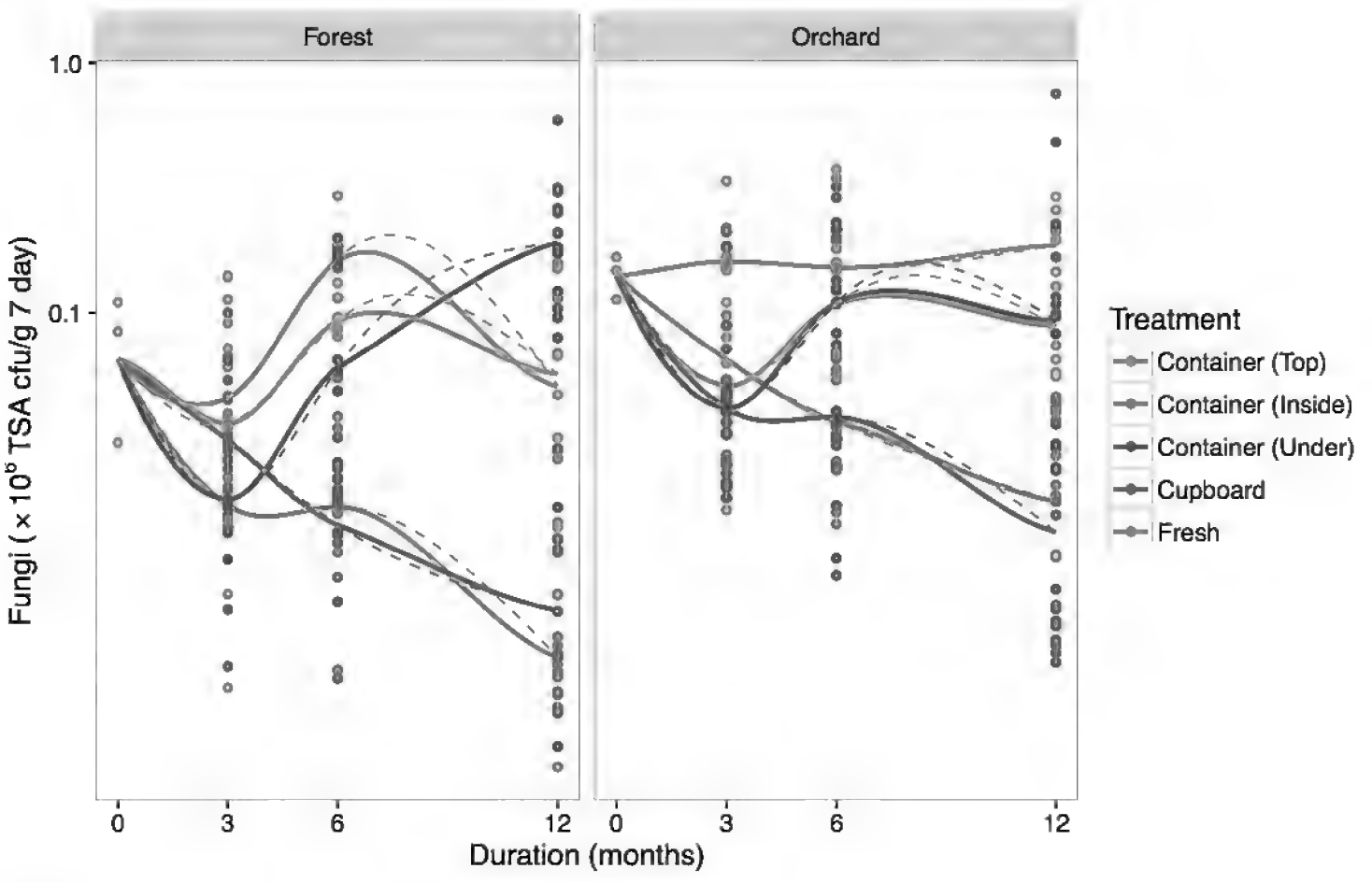

Figure 3. Scatterplot of mean fungus counts (cfu/g) at 0, 3, 6 and 12 months measured in soil recovered from on, in and under four sea containers and inside four cupboards. Solid lines show the smoothed means and dashed lines predictions from the preferred model.

after 12 months, which both descended to about a tenth of the original counts. *Pseudomonas* spp. counts in soil located under containers, followed by soil inside containers, were intermediate between fresh soil and cupboard soil except at 6-month period. Counts in soil located on top of containers showed a temporary plateau at 6 months.

Fungi

Modelled and mean observed changes in fungus cfu/g across time are presented in Figure 3. Fungus counts in soil on top of and under containers was comparable to fresh soil, with little mortality occurring during the experiment. Fungi from soil held inside either the cupboard or the container showed the highest mortality after 6 months, with counts dropping about ten-fold over the 12 months. The orchard site had more fungi than the forest, but in the absence of replication we cannot ascribe this difference to site.

Nematodes

Modelled and mean observed changes in nematode counts over time are presented in Figure 4. In the day zero sample, there were means of 22 and 33 total nematodes/g of

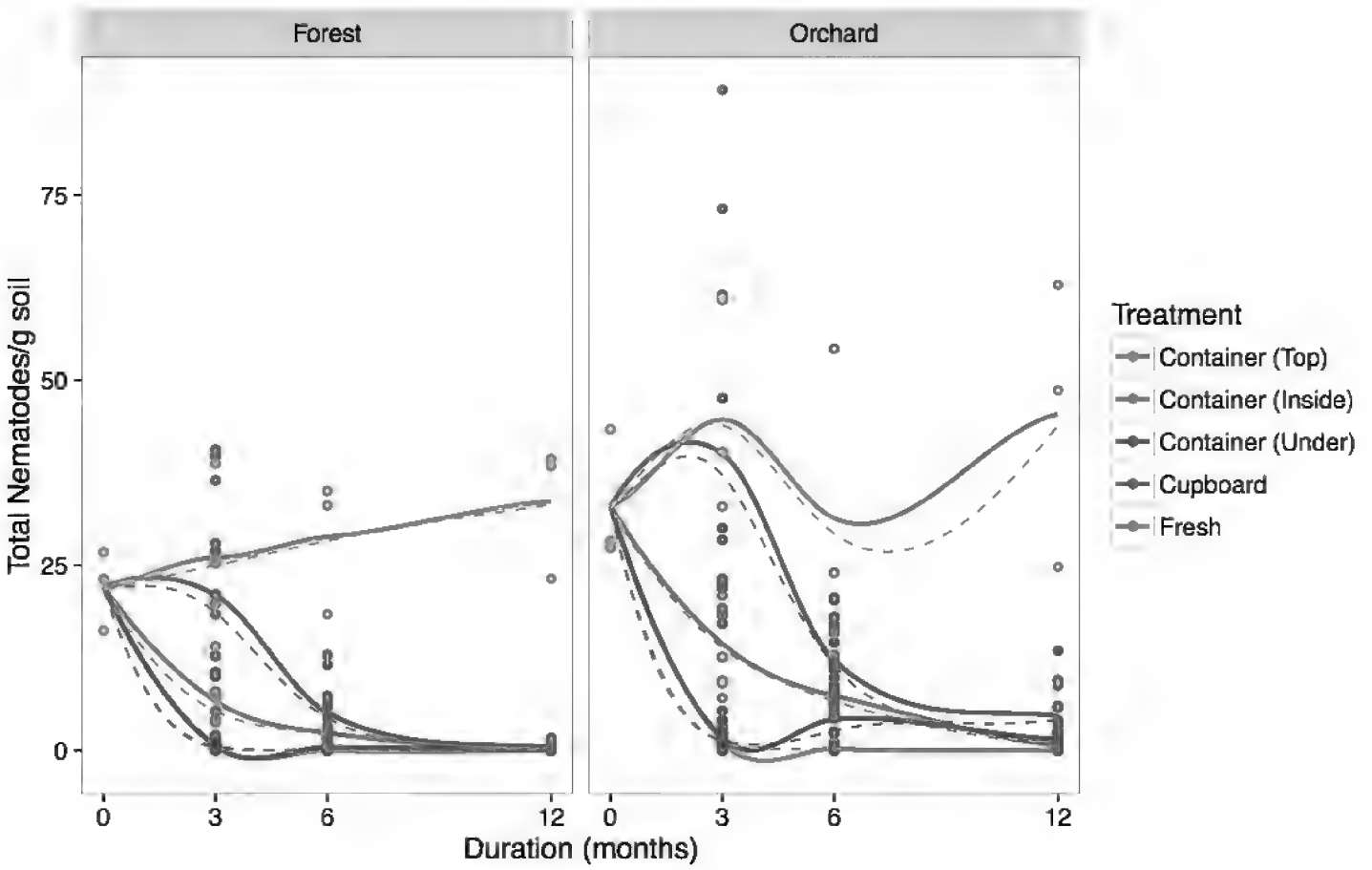

Figure 4. Scatterplot showing changes in mean total nematodes/ g soil at 0, 3, 6 and 12 months measured in soil recovered from on, in and under four sea containers and inside four cupboards. Solid lines show the smoothed means and dashed lines predictions from the preferred model.

soil for forest and orchard soil, respectively. Counts declined in all storage treatments over time. Fresh soil collected at 3, 6 and 12 months had higher counts than stored soil. With stored soil, nematode counts were higher in orchard soil than forest soil, with survival in cupboards generally higher than in sea containers over the 12 months.

Mortality was highest in soil located either on or under the sea containers, with approximately 7.5- and 12-fold declines in total nematodes for the orchard and forest soil, respectively, after 3 months. Thereafter, total nematode numbers were negligible for the rest of the experiment. By comparison, for soil held inside the sea containers, there was only a 2- and 3-fold decline in nematode numbers after 3 months for the orchard and forest soil, respectively. At 6 months, there were 5- and 6-fold declines in numbers for the orchard and forest soils, respectively. Total nematode counts were close to zero after 12 months.

For soil held in cupboards, nematode counts did not change for the first 3 months, but after 6 months there were 4- and 5-fold declines in the orchard and forest soil, respectively. At 12 months, this decline had increased, to a 55-fold decrease in forest soil compared to 7-fold decrease in orchard soil.

Plant-parasitic nematodes

PPN recovered were from the genera *Pratylenchus*, *Paratylenchus*, *Heterodera* and *Helicotylenchus*, and the family Criconematidae. There was a higher diversity of

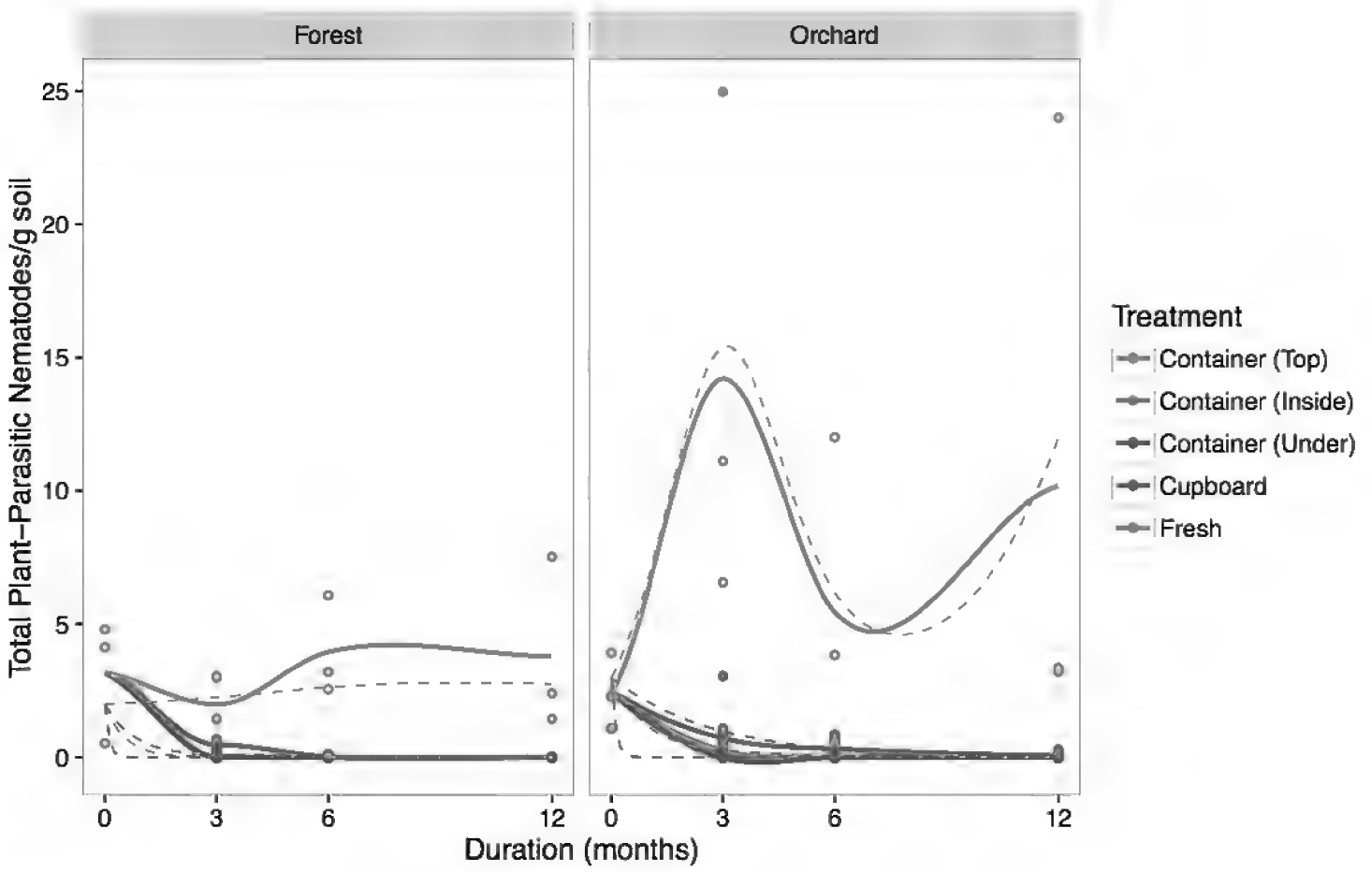

Figure 5. Scatterplot showing changes in mean plant-parasitic nematodes/ g soil at 0, 3, 6 and 12 months measured in soil recovered from on, in and under four sea containers and inside four cupboards. Solid lines show the smoothed means and dashed lines predictions from the preferred model.

plant parasitic nematodes in the forest soil (*Pratylenchus*, *Paratylenchus*, *Globodera*, *Helicotylenchus, Rotylenchus* and Criconematidae) than the orchard soil (*Pratylenchus, Paratylenchus* and *Heterodera* spp.). In addition, many plant parasitic species from the forest soil could only be identified to genus. Changes in the count of PPN across time are presented in Figure 5. In the day zero sample, there were means of 3 and 2.4 PPN/ g of soil for forest and orchard soil, respectively. Fresh soil collected from the source sites at 3, 6 and 12 months had higher plant parasitic nematode counts than stored soil (Figure 5). As observed for total nematodes, PPN declined rapidly in stored soil, with the rate of decline highest in soil situated on or under sea containers, and lowest in soil in cupboards. PPN were not detected in soil positioned on or under sea containers from 3 months onwards. Survival was highest in soil in cupboards, and better in orchard than forest soil. PPN were present in orchard soil after 12 months, with a 40-fold decline over that period. In comparison, in forest soil, PPN were not detected after 6 months, showing a 122-fold decline over that period. There was a decline in PPN bio-diversity for all storage treatments over time, both relative to the original soil samples and compared with the two sites were resampled at 3, 6 and 12 months. Mean number of taxa found in soil from the forest and orchard over the 12 months averaged 3.9 ± 0.23 and 2.0 ± 0.12, respectively. By comparison, in stored soil, the mean number of taxa in soil where PPN were present, averaged (± SEM) 1.5 ± 0.29 and 1.1 ± 0.08 after 3 months for cupboard and sea container soil, respectively. By 6 months,

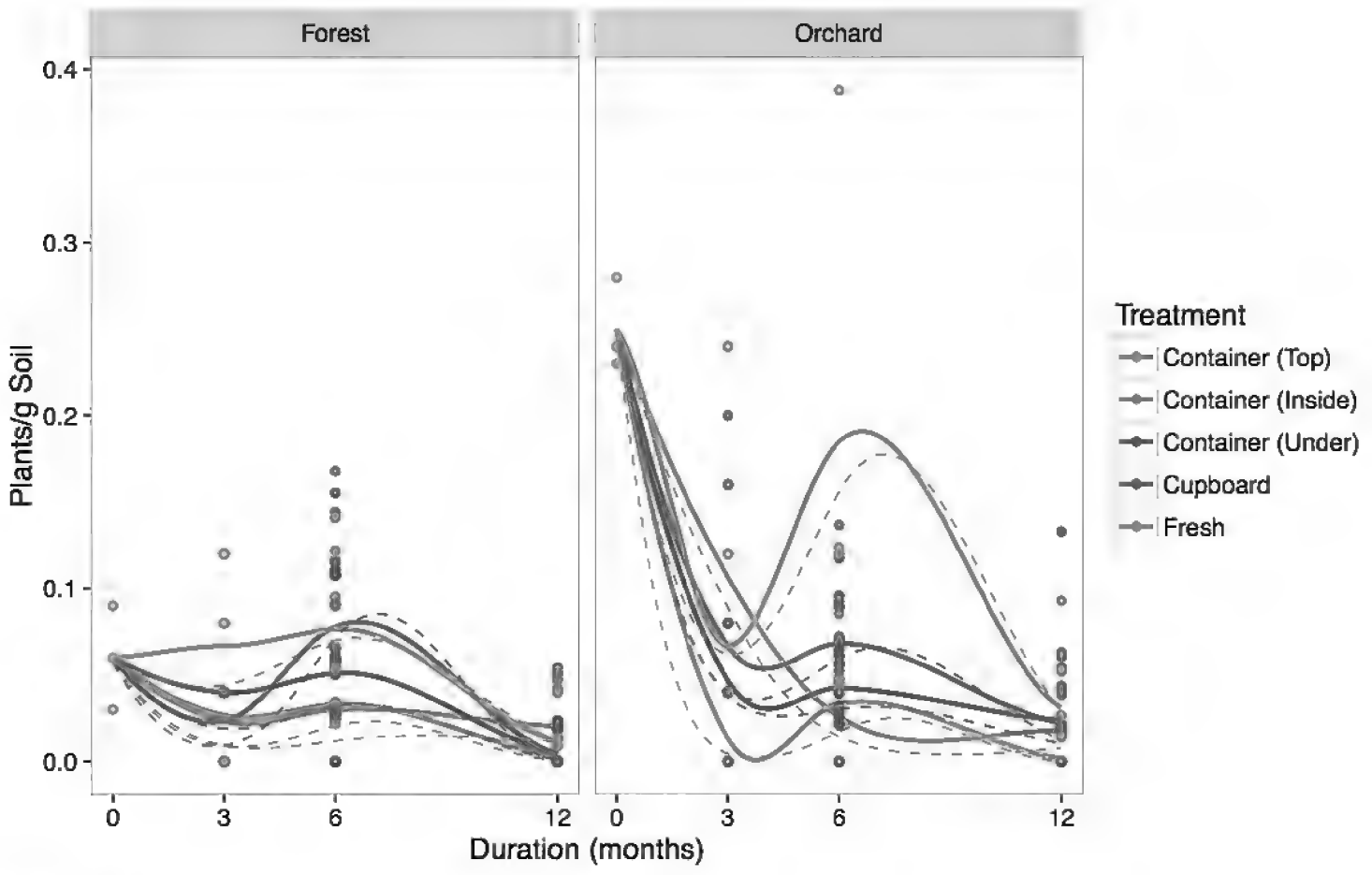

Figure 6. Scatterplot showing changes in mean plants/g soil grown from soil collected at 0, 3, 6 and 12 months recovered from on, in and under four sea containers and inside four cupboards. Solid lines show the smoothed means and dashed lines predictions from the preferred model.

the mean number of taxa recovered from cupboard and sea container soil was 1.1 ± 0.07 and 1.0 ± 0.0 after 6 months and 1.0 ± 0.0 and 0 after 12 months, respectively. *Pratylenchus* species were the dominant genus (84%) recovered from stored soil from 3-12 months irrespective of location.

Plants

Overall, the orchard soil had more seeds/g soil than forest soil, but in the absence of replication we cannot ascribe this difference to site. The number of plant species per soil was similar, with 29 and 38 species in the forest and orchard soils, respectively, but the composition of plant species between the two sites was markedly different. In the forest soil, 13 native and 16 exotic species were found, while in the orchard soil one native and 37 exotic species were present. Only 13 species were common to both sites (Suppl. material 2), all exotic invasive weed species, with *Solanum nigrum* L. (black nightshade) being the most frequently recorded species in both soils.

Soil in relatively exposed locations on or under sea containers did not recruit significantly more new plant species than soil in relatively protected locations inside cupboards or sea containers (data not shown).

Changes in plant counts per g of soil over time are presented in Figure 6. In the day-zero sample, there were means of 0.3 and 0.1 plants/g of soil for forest and orchard soil, respectively. Fresh soil had higher total plant numbers and plants/g of soil than stored soil. The number of plants that germinated declined significantly after 12 months for soil in all storage treatments from both sites. Soil stored on top of containers showed the greatest decline, while soil stored elsewhere showed declines that were similar to one another. Plant species count (not shown) showed a similar response, regardless of whether raw counts were used, or species per g of soil.

Soil chemistry

The forest soil is a Taitapu deep silt loam described as a recent gley soil, while the orchard soil is a Wakanui deep silt loam and described as a mottled immature pallic soil. Both soils comprise 15-35% clay. Based on the USDA Soil Series Classification, the Taitapu soil is a Typic Fluvaquent and the Wakanui soil an Udic Dystocrept. They had similar pH, but P, K, total phosphorus and Cu were higher in the orchard soil, while Fe was higher in the forest soil. Soil chemistry had no significant effect on counts of any taxa. Details of the soil chemical analysis are in the Suppl. material 3.

Temperature

Temperatures (Figure 7) varied with season and treatment, being lowest during winter (June-August) and highest in summer (December-February). Throughout the year, the lowest minimum temperature was recorded on top of containers (-6.5 °C), with a maximum temperature of 30.7 °C. The highest temperature fluctuations and maximum temperatures occurred inside containers with a range of -5.3 to 36.9 °C. Temperatures under containers were consistently bounded by temperatures in other treatments and ranged from -5.0 to 29.1. Temperatures varied least inside cupboards (0.8-26.0 °C).

Synthesis

Figure 8 summarises how relative numbers of each taxon changed with storage duration in each storage location. Nematodes, plants and *Pseudomonas* spp. bacteria exhibited one set of characteristic responses to the treatments, and fungi and other bacteria exhibited another. When stored in exposed locations, survival of nematodes, plants and *Pseudomonas* spp. bacteria rapidly declined to less than c. 25% of the original numbers after 3 months and less than c. 10% after 12 months (Figure 8). When stored in protected locations, the decline to c. 25% of the original numbers took c. 6 months rather than three (Figure 8). Fungi and bacteria (other than *Pseudomonas* spp.) showed

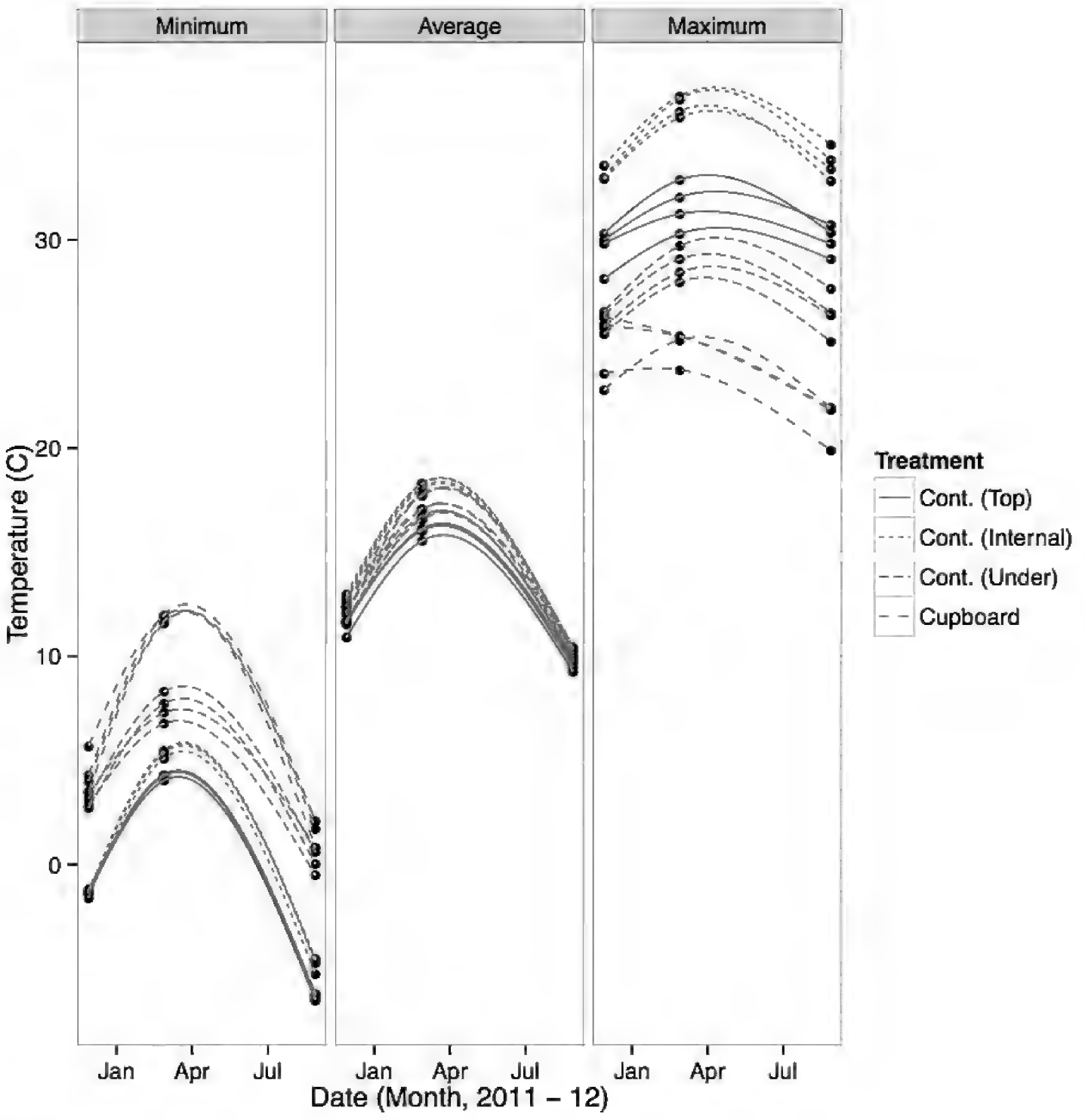

Figure 7. The minimum, average and maximum temperatures recorded on, in and under four sea containers and inside four cupboards over a 12 month period. Lines of the same colour represent different replicates of the same treatment.

the same patterns as the other taxa when stored in interior locations. However, when stored on top of or under containers, populations of fungi and non-*Pseudomonas* bacteria fluctuated widely with storage time, though only fungi stored under containers remained above their original levels after 12 months.

Discussion

This research is the first to estimate the effects of storage time and environmental exposure on soil-borne taxa that could be biosecurity hazards. The results showed clear patterns that

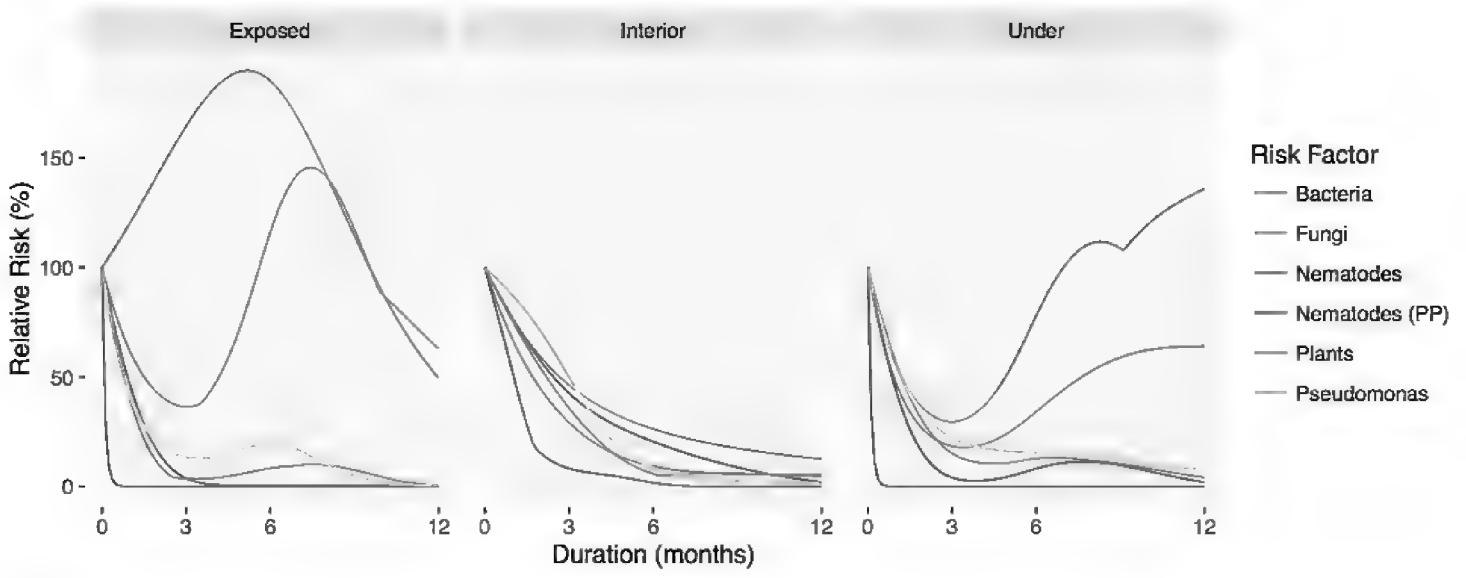

Figure 8. Changes in relative amounts of biota against duration, by storage location. Where the relative amount differed by soil origin, the maximum was chosen. In the legend, 'Nematodes (PP)' represents plant parasitic nematodes.

should prove useful for targeting management resources at soil contaminants that are the most hazardous, particularly when combined with results of other studies.

Unlike other taxa, fungi and non-*Pseudomonas* bacteria in soil stored in exposed locations showed large population fluctuations during storage. It is uncertain if these fluctuations were due to growth and mortality of the fungi and bacteria originally present in the samples – perhaps associated with fluctuations in populations of algae and other food substrates – or if the taxa concerned colonised the soil after it was originally collected (S. Wakelin, AgResearch, personal comm.). Molecular analysis of the samples we retained from this experiment that are currently stored at -80 °C might help to clarify which of these processes was most important. This issue is pertinent because, if fungi and bacteria present in transported soil tend to be recent colonisers, then they are more likely to be local rather than introduced taxa, and often may not be biosecurity hazards.

We expected soil stored in exposed locations would be invaded by additional organisms such as windborne seeds as the experiment proceeded, but the results showed both that soil stored in exposed locations contained similar densities of viable seeds to soil stored in protected locations, and that seed viability declined with storage duration. It is possible the similarity between the exposed and protected treatments was an artefact of our experimental setup: For example, our sea containers were situated on a paved surface, approximately 20 m from the nearest vegetation, thus reducing propagule pressure; and the trays that contained the soil had 50 mm high rims which may have impeded seed entry. However, it is more likely this result is indicative of real-world soil contaminants on sea containers because it is similar to seed count data obtained from a recent study of soil intercepted from sea containers at New Zealand ports (McNeill et al. in prep.). Moreover, our experimental result also matched the 0.03 seeds that germinated per gram of soil intercepted from construction vehicles in Antarctica (Hughes et al. 2010). Thus, we tentatively conclude that soil contaminants on sea containers represent small, difficult-to-hit targets for windborne seeds, so seed

counts per gram of soil will seldom increase during transport, while seed viability will generally decline. It is even possible that in some circumstances seed counts per gram of soil will also decline with transport duration due to granivory by birds, rodents and arthropods.

The organisms contained in soil from each of our sources (orchard and forest) showed nearly identical responses to storage duration and location. Although soil source was unreplicated, these results were consistent with our expectation, based on previous observations (Chu et al. 2010, Decaens 2010, Kuske et al. 1997, McNeill et al. 2011), that higher taxonomic groups will usually be shared between soil sources, and those obtained from different locations will show similar responses to storage duration and location.

It is interesting to compare our results from fresh orchard and forest soil to those from a study of soil on international air passengers' footwear (McNeill et al. 2011). Air passengers' footwear gave similar counts per gram of soil for bacteria and nematodes, and even higher values for seeds and fungi. This is consistent with short transport duration (low soil age), low environmental exposure, and relatively high risks from soil transported on airline pathways.

In general, our results supported the hypotheses that some soil organisms transported on the external surfaces of shipping containers will suffer high mortality rates due to environmental exposure (Marshall and Varney 2000), and that duration of soil transport will negatively influence soil organism survival (McNeill et al. 2011). This suggests there is potential to rank risks presented by soil on different pathways by comparing environmental exposure and transport duration between pathways, thus enabling management to mitigate the highest risks. Risks will also vary depending on the diversity and also the life stages of taxa (e.g. cysts, eggs and seeds/spores in dormant stages) vectored with soil. Also the risks are likely to vary between taxa as some genera and/or species may have better survival rates than others. To further develop and test this approach, we need measurements of the incidence and abundance of soil organisms from a greater range of pathways (including conveyances), and better pathway-specific estimates of soil age.

An important aspect of risk analysis relevant to soil contaminants that has seldom been studied is between-pathway variation in the likelihood that organisms vectored by soil will be transported to habitats suitable for their establishment. Soil on plants imported for planting would presumably present a particularly high risk in this regard because environmental exposure and transport duration will likely be low and, unless the soil is removed and quarantined, there will be a high probability that IAS will be transferred with the plants to habitats suitable for organism establishment (Miglionini et al. 2015). Organisms vectored in soil adhering to footwear might also have high potential for transfer to suitable habitats through treading in unpaved areas, while, at first glance, establishment opportunities for organisms in sea container soil perhaps seem less. Proper investigations of such possibilities would contribute greatly to improved management of biosecurity risks from transported soil.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contribution

MM: Developed the research concept, led and carried out the sampling, contributed to manuscript writing. CP: Developed the research concept, contributed to sampling and manuscript writing, AR: Analyzed data and contributed to manuscript writing. LA: Carried out the extraction and identification of nematodes and contributed to manuscript writing, NR: Carried out the extraction and identification of Pseudomonas and contributed to manuscript writing, SY: Carried out the isolation and countijng of bacteria and fungi and contributed to manuscript writing, CD: Carried out the rearing and identification of plants and contributed to manuscript writing; TJ: Assisted in rearing and identification of plants, NB: Assisted in identification of nematodes

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Supplementary material 1

Description of the data analysis methods and results

Authors: Mark McNeill, Craig Phillips, Andrew Robinson, Lee Aalders, Nicky Richards, Sandra Young, Claire Dowsett, Trevor James, Nigel Bell

Data type: data analysis methods and results

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Supplementary material 2

Plant species common to both soil sources

Authors: Mark McNeill, Craig Phillips, Andrew Robinson, Lee Aalders, Nicky Richards, Sandra Young, Claire Dowsett, Trevor James, Nigel Bell

Data type: species description

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Supplementary material 3

Soil chemistry of the two soil sources

Authors: Mark McNeill, Craig Phillips, Andrew Robinson, Lee Aalders, Nicky Richards, Sandra Young, Claire Dowsett, Trevor James, Nigel Bell

Data type: soil chemistry

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